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Synergistic interactions between kainate and mGlu receptors regulate bouton Ca^{2+} signalling and mossy fibre LTP

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It is currently unknown why glutamatergic presynaptic terminals express multiple types of glutamate receptors. We have addressed this question by studying both acute and long-term regulation of mossy fibre function in the hippocampus. We find that inhibition of both $mGlu_1$ and $mGlu_5$ receptors together can block the induction of mossy fibre LTP. Furthermore, mossy fibre LTP can be induced by the pharmacological activation of either $mGlu_1$ or $mGlu_5$ receptors, provided that kainate receptors are also stimulated. Like conventional mossy fibre LTP, chemically-induced mossy fibre LTP (chem-LTPm) depends on Ca^{2+} release from intracellular stores and the activation of PKA. Similar synergistic interactions between mGlu receptors and kainate receptors were observed at the level of Ca^{2+} signalling in individual giant mossy fibre boutons. Thus three distinct glutamate receptors interact, in both an AND and OR gate fashion, to regulate both immediate and long-term presynaptic function in the brain.

The most extensively studied form of synaptic plasticity in the CNS is long-term potentiation (LTP) of glutamatergic synaptic transmission in the hippocampus[1]. Two distinct forms of LTP have been described in the vertebrate CNS, based on whether their induction does[2] or does not[3] require the synaptic activation of N-methyl-D-aspartate (NMDA) receptors. The best characterised form of NMDA receptor-independent LTP is at mossy fibre synapses in the hippocampus. However, considerable controversy still surrounds the mechanism of induction of this form of LTP. Originally it was believed that the induction of mossy fibre LTP was independent of the activation of ionotropic glutamate receptors[4]. However, it was then found that metabotropic glutamate (mGlu) receptors (mGluRs) are involved in the induction of mossy fibre LTP[5–9], although not invariably so[10,11]. Subsequently, a role for kainate receptors (KARs) in the induction of mossy fibre LTP was identified[12–17].

These findings raise several fundamental questions. First, what are the subtypes of mGluRs and KARs that are involved in mossy fibre LTP? Second, is activation of these subtypes, either in isolation or in combination, sufficient to induce mossy fibre LTP or is the activation of other receptors also required? Third, since mossy fibre LTP is generally believed to be induced presynaptically, does the activation of mGluRs and KARs regulate Ca^{2+} signalling in mossy fibre boutons and, if so, how do they interact? To address these issues we have, firstly, studied mossy fibre LTP in a slice preparation in which we have previously identified roles for mGluRs[5] and KARs[12] and, secondly, studied Ca^{2+} signalling in individual mossy fibres, using 2-photon microscopy, as described previously[18,19].

We demonstrate that activation of group I mGluRs is required for the induction of mossy fibre LTP. Surprisingly, however, either mGlu1 or mGlu5 receptors can serve this role, since antagonism of both subtypes together is required for inhibition of LTP. This is a rare example of two subtypes playing interchangeable roles in the regulation of synaptic function. However, activation of group I mGluRs was not sufficient for the induction of LTP suggesting that additional receptors may need to be co-activated. Interestingly, if either $mGlu_1$ or $mGlu_5$ receptors are activated in conjunction with KARs, using the GluK1 selective agonist ATPA, then a robust form of LTP is induced. This novel form of chem-LTP can be completely prevented by depletion of Ca^{2+} stores with ryanodine. Significantly, these effects were mirrored by a similar regulation of Ca^{2+} in individual mossy fibre giant boutons. Thus, inhibition of either group I mGluRs or KARs reduced the Ca^{2+} transient evoked by a brief train of action potentials evoked in a granule cell. Furthermore, co-activation of group I mGluRs and KARs resulted in a long-term regulation of Ca^{2+} in mossy fibre boutons, manifested both as a broadening of the action potential-evoked Ca^{2+} transient and an elevation in basal Ca^{2+}, effects that were prevented by ryanodine. The observation

that three receptor subtypes interact in an unusual manner (involving activation of KARs AND either $mGlu_1$ OR $mGlu_5$ receptors) in two aspects of mossy fibre function (LTP and Ca^{2+} signalling in giant boutons) suggests a causal relationship between these two effects. We therefore propose that one form of mossy fibre LTP involves a persistent regulation of Ca^{2+} signalling in giant mossy fibre boutons and that this is triggered by the simultaneous activation of mGluRs and KARs.

Results

Antagonism of both $mGlu_1$ and $mGlu_5$ receptors is required to block the induction of mossy fibre LTP. MCPG (α-methyl-4-carboxyphenylglycine) is a broad spectrum mGlu receptor antagonist which is roughly equipotent at $mGlu_1$, $mGlu_2$, $mGlu_3$, $mGlu_5$ and $mGlu_8$ receptors[20]. Previously we reported that, at a concentration of 200 μM, (S)-MCPG can fully block the induction of NMDA receptor-independent LTP in the CA3 region of rat hippocampal

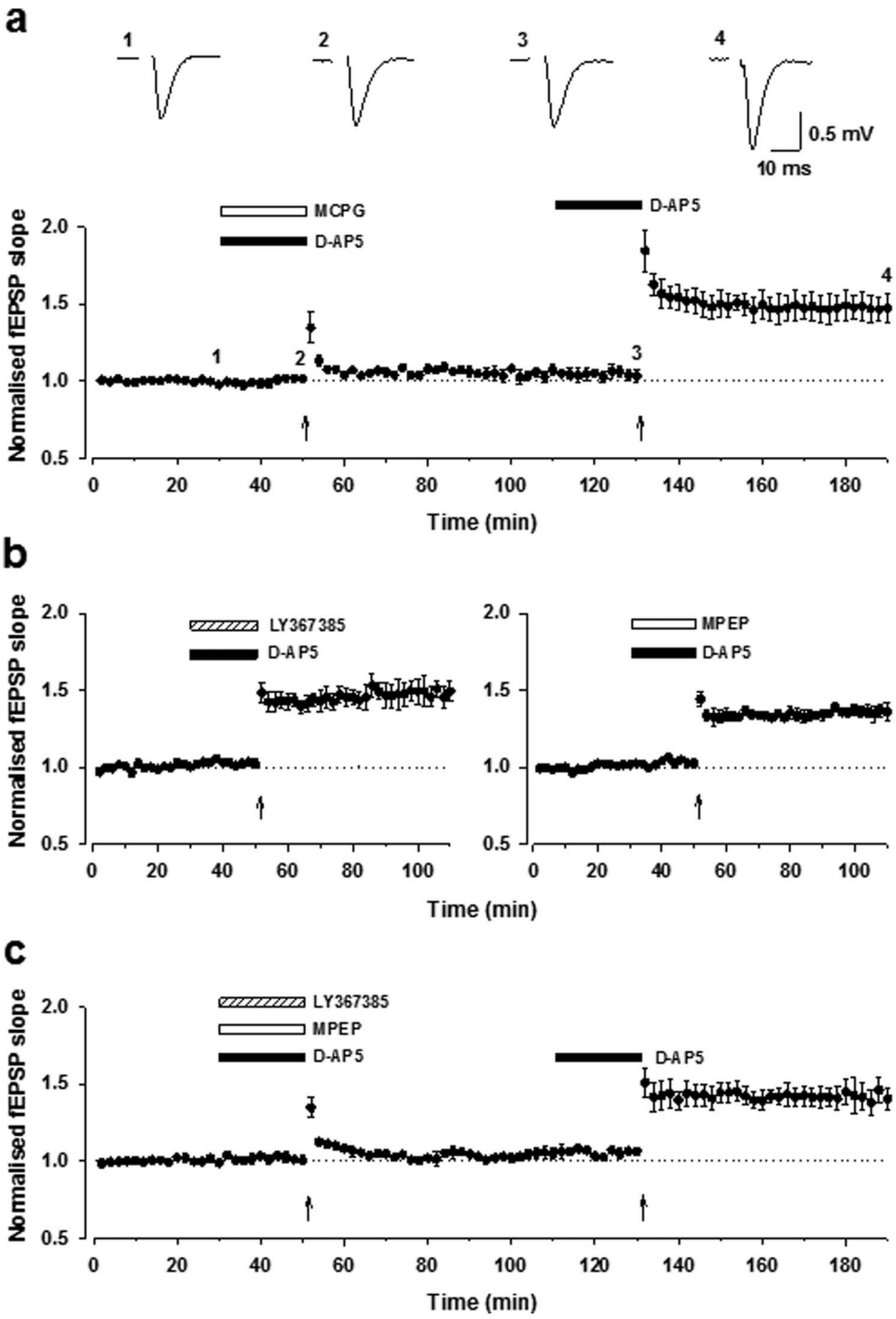

Figure 1 | Antagonism of both $mGlu_1$ and $mGlu_5$ receptors inhibits the induction of mossy fibre LTP. (a) The graph represents pooled data from 4 experiments to show that MCPG (200 μM) always blocked the induction of LTP (100 Hz, 1 s) in a reversible manner. In these, and subsequent experiments, D-AP5 (50 μM) was always present during each tetanus to ensure that only NMDA receptor-independent LTP was studied. Bars indicate the exposure time to various compounds. Insets at the top are representative fEPSPs taken at the indicated time on the plot. (b) Pooled data from 4 experiments showing the lack of effect of the selective $mGlu_1$ antagonist LY367385 (30 μM) [left panel] and $mGlu_5$ antagonist MPEP (30 μM) [right panel]. (c) Pooled data from 4 experiments showing reversible block of the induction of mossy fibre LTP by co-application of LY367385 (3 μM) plus MPEP (3 μM).

slices[5]. The ability of MCPG to block the induction of mossy fibre LTP has been verified in some[7,21] but not all[11] subsequent investigations. Here we confirm that, in the presence of 200 μM (S)-MCPG (and 50 μM D-AP5 to additionally block NMDA receptors), high frequency stimulation (100 pulses at 100 Hz, test intensity) consistently failed to induce LTP (60 min post-induction: 103 ± 3%; $P > 0.05$). Following a 60 min washout period, the same induction protocol then induced LTP of mossy fibre responses (147 ± 9%; $n = 4$; $P < 0.005$; Fig. 1a).

To investigate the mGlu receptor subtype(s) involved in the induction of mossy fibre LTP we used an $mGlu_1$ selective antagonist, LY367385 and an $mGlu_5$ selective antagonist, MPEP. Neither compound affected the induction of LTP even when applied at high concentrations (30 μM), well in excess of that required to block their respective receptors. Thus, the respective levels of LTP induced under these conditions were 149 ± 7%; $n = 4$ and 136 ± 6%; $n = 4$, respectively (Fig. 1b). Since MCPG is able to block both $mGlu_1$ and $mGlu_5$ receptors we tested a combination of LY367385 and MPEP, to selectively block just these two subtypes. We found that this combination fully blocked the induction of LTP, even when the antagonists were both applied at 3 μM, a ten-fold lower concentration that that which was ineffective when each was applied alone (60 min post-induction: 103 ± 3%; following a 60 min washout 140 ± 7%; $n = 4$; $P < 0.005$; Fig. 1c). These results show that mossy fibre LTP requires activation of either $mGlu_1$ or $mGlu_5$ receptors, such that inhibition of both is required to block the induction of LTP.

Synergistic interaction between group I mGlu receptors and kainate receptors can induce synaptic potentiation at mossy fibres. We wondered whether activation of mGlu receptors was not only necessary but also sufficient for mossy fibre LTP. We therefore applied the group I selective agonist DHPG to see if we could induce LTP. We found that 3 μM DHPG had no effect (Fig. 2a), whilst higher concentrations resulted in a transient depression of synaptic transmission (data not shown). These observations suggest that activation of group I mGluRs is not sufficient to induce LTP at mossy fibre synapses.

Previously, we have reported that mossy fibre LTP can also be blocked by KAR antagonists, including those that selectively target the GluK1 subtype[18,19,22,23]. We therefore attempted to induce LTP by the co-application of DHPG with the GluK1 selective agonist ATPA[24]. ATPA (1 μM) applied alone had no effect (Fig. 2a). However, the co-application of ATPA (1 μM) and DHPG (3 μM) elicited a robust long-lasting potentiation (140 ± 10%; $n = 5$; $P < 0.005$; Fig. 2a), which we have termed chemically-induced mossy fibre LTP (chem-LTPm).

The finding that it was necessary to block both $mGlu_1$ and $mGlu_5$ receptors to prevent the induction of mossy fibre LTP suggests that activation of either group I mGlu receptor subtype alone may be

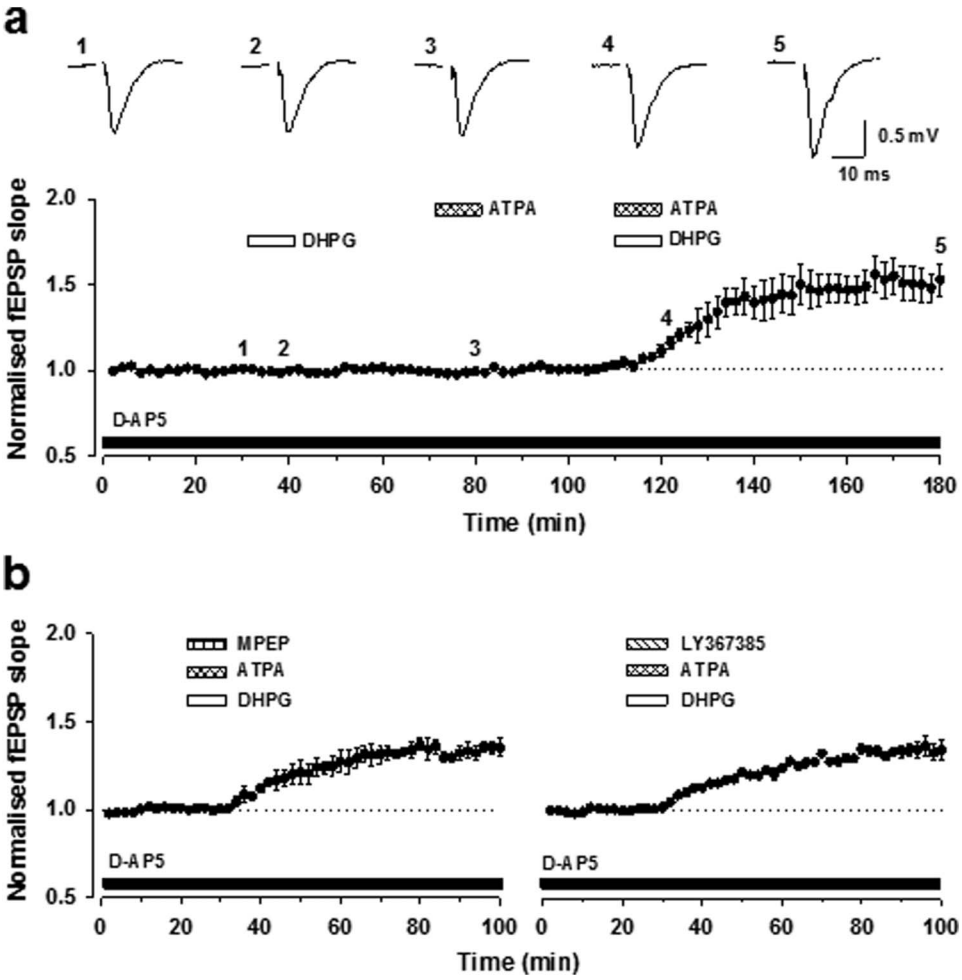

Figure 2 | Chemically-induced mossy fibre LTP. (a) Pooled data from 5 experiments showing that co-application of ATPA (1 μM) plus DHPG (3 μM), but neither agonist alone, induces a potentiation of mossy fibre synaptic transmission. In these and subsequent experiments D-AP5 (50 μM) was present throughout. Insets at the top are representative EPSPs taken at the indicated time on the plot. (b) Chem-LTPm can be induced by activation of GluK1-containing KARs plus activation of either $mGlu_1$ receptor subtype. Both panels illustrate pooled data from 4 experiments that show synaptic potentiation induced by ATPA (1 μM) plus DHPG (3 μM), in the presence of MPEP (30 μM) (left panel) or in the presence of LY367385 (30 μM) (right panel).

sufficient to induce LTP, in conjunction with activation of GluK1-containing KARs. We therefore applied ATPA, to activate GluK1, plus DHPG either in the presence of MPEP (to selectively activate $mGlu_1$) or in the presence of LY367385 (to selectively activate $mGlu_5$) and found that either treatment was sufficient to induce synaptic potentiation (135 ± 5%; $n = 4$; $P < 0.005$ and 134 ± 5%; $n = 4$; $P < 0.005$, respectively; Fig. 2b). Therefore it can be concluded that activation of KARs AND [$mGlu_1$ OR $mGlu_5$ receptors] is sufficient for the induction of chem-LTPm.

To establish whether chem-LTPm shared similar mechanism as synaptically-induced mossy fibre LTP we tested its sensitivity to KT5720, to inhibit PKA, and ryanodine, to inhibit Ca^{2+} stores, since these agents are known to block synaptically-induced mossy fibre LTP[25,26]. Both treatments fully blocked chem-LTPm in a reversible manner (3 μM KT5720: 103 ± 2%, $n = 4$; Fig. 3a; 10 μM ryanodine: 103 ± 3%; $n = 4$; Fig. 3b).

Mossy fibre LTP is widely believed to be both induced and expressed presynaptically. Therefore, to home in on the cellular mechanisms engaged at single presynaptic terminals we used a technique that allows calcium signalling to be imaged in individual mossy fibre boutons[18,19]. Single granule cells were loaded with both a morphological marker (Alexa 594) and a Ca^{2+} sensitive dye (Fluo-4) to enable the mossy fibre axon to be traced into the CA3 region and the regulation of Ca^{2+} signalling by glutamate receptors to be unambiguously studied in single mossy fibre boutons (Fig. 4a and 4b). Five action potentials evoked in the granule cell at 20 Hz resulted in a readily discernible Ca^{2+} transient in the giant bouton (Fig. 4a). The GluK1-selective compound LY382884 (10 μM) significantly reduced calcium entry into presynaptic terminals (peak ΔF/F was reduced to 70 ± 1%; $n = 3$; Fig. 4c). This finding is consistent with previous data obtained in younger animals using a different KAR antagonist, ACET[19]. Co-application of MPEP (3 μM) and LY367385 (3 μM) to antagonise both $mGlu_1$ and $mGlu_5$ receptors also decreased calcium entry into individual presynaptic boutons (peak ΔF/F was reduced to 64 ± 7%; $n = 4$; Fig. 4d). The effect of these antagonists was most pronounced on the Ca^{2+} transients evoked by the latter action potentials within the train. This, as well as the magnitude of the effects, are entirely consistent with previous studies[18,19] and probably reflect the autoreceptor function of these receptors[26].

The close correlation between the ability of the glutamate receptor antagonists to block the induction of mossy fibre LTP and to depress a component of the Ca^{2+} transient in mossy fibre boutons, evoked by action potentials elicited in granule cells, suggests that these two processes are causally linked. In which case, it follows that chem-LTPm might be associated with alterations in mossy fibre Ca^{2+} signalling. To address this issue, single action potentials were evoked at a frequency of 0.033Hz (Fig. 5a) and then ATPA (1 μM) and DHPG (3 μM) were co-applied at a concentration that elicits chem-LTPm (Fig. 5c). This treatment had two effects: it induced a sustained rise in intracellular calcium (basal calcium increase: 99 ± 10%; $n = 3$; Fig 5d) and resulted in a broader Ca^{2+} transient in response to single action potentials (Fig. 5c).

In interleaved experiments, we applied ryanodine (10 μM) and this prevented both effects. Co-application of ATPA and DHPG in the presence of ryanodine did not alter basal calcium levels (basal calcium was 115 ± 6%; $n = 3$; Fig. 6b) neither did it affect the rate of decay of the calcium transient (Fig. 6d). However, when ryanodine was washed out and DHPG plus ATPA were re-applied, the effects were again observed. The chem-LTPm protocol increased basal Ca^{2+} (150 ± 7%; $n = 3$; Fig. 6c) following washout of ryanodine , and the rate of decay of fluorescence transients was slower compared to control conditions. The signal remaining at t* (1.4 s after peak) was 17 ± 3% in the presence of ATPA+DHPG compared to 44 ± 5% in control conditions (Fig. 6d). Therefore, the changes in

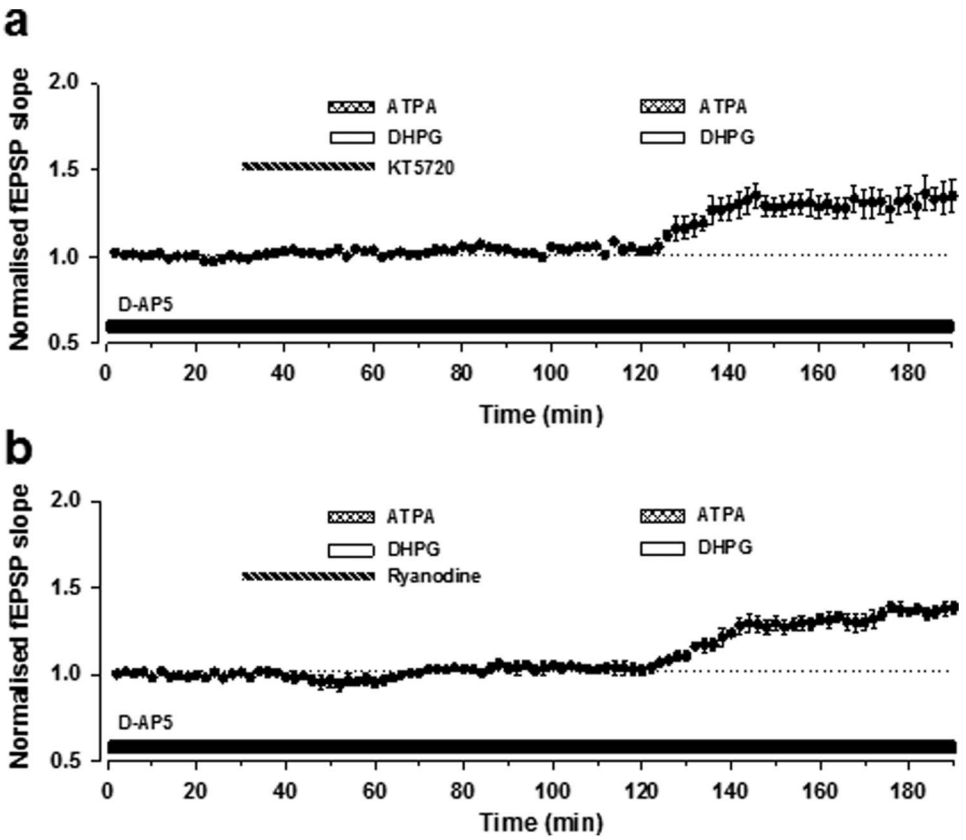

Figure 3 | Properties of chem-LTPm. (a) Chem-LTPm involves activation of PKA. Pooled data from 4 experiments to illustrate that KT5720 (3 μM) reversibly inhibits synaptic potentiation induced by ATPA (1 μM) plus DHPG (3 μM). (b) Chem-LTPm involves Ca^{2+} release from intracellular stores. Pooled data from 4 experiments to illustrate that ryanodine (10 μM) reversibly inhibits synaptic potentiation induced by ATPA (1 μM) plus DHPG (3 μM).

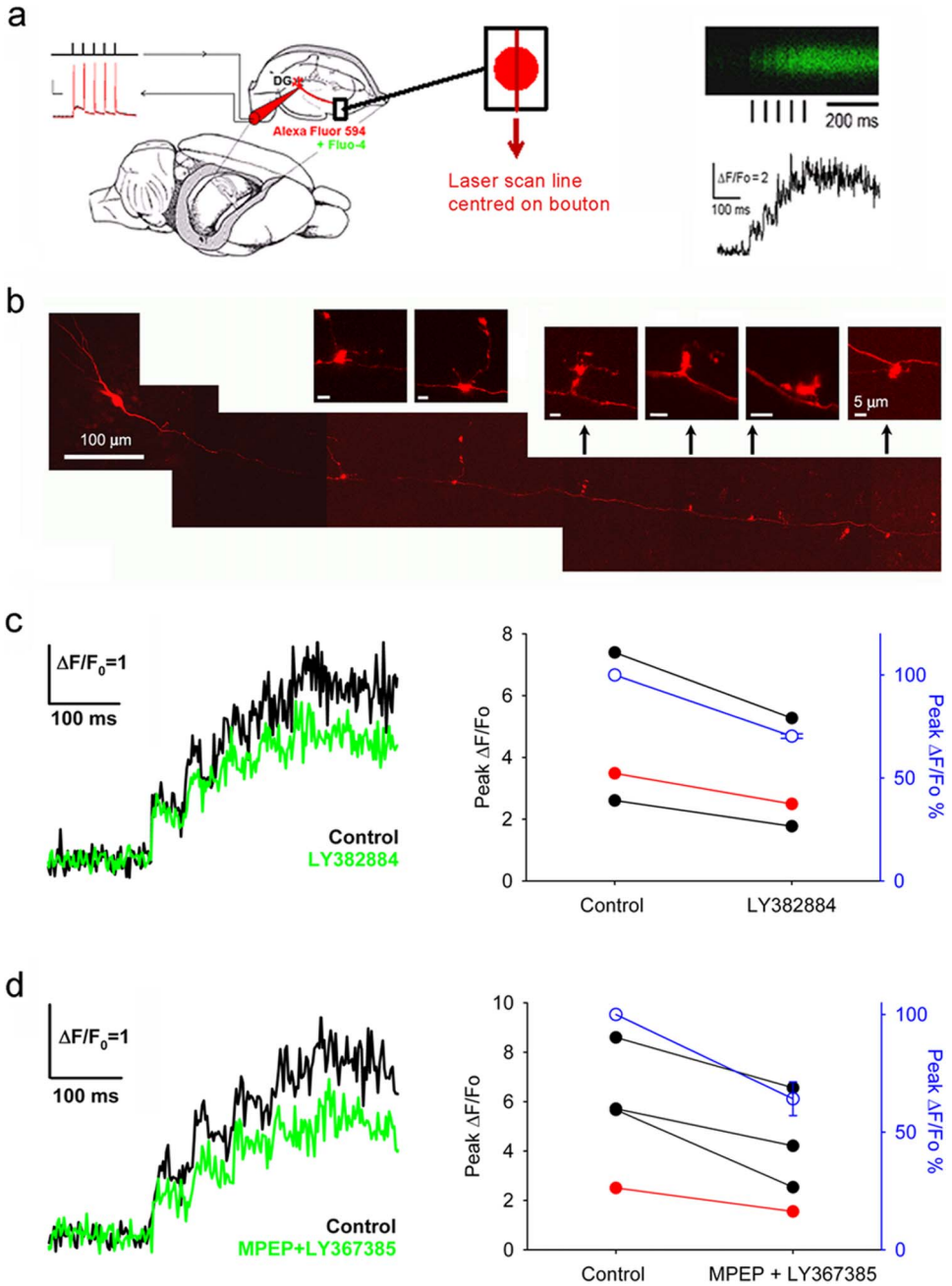

Figure 4 | Both GluK1-containing KARs and group I mGlu receptors regulate Ca^{2+} signalling at individual mossy fibre boutons. (a) Schematic illustration of technique (see methods for details) and typical fluorescence transient evoked by delivering a train of action potentials (5 at 20 Hz). (b) Representative single mossy fibre traced into CA3. Giant mossy fibre boutons were identified by their characteristic size (3 – 8 µm) and filopodial extensions. (c) Typical fluorescence transients recorded in line scanning mode before (black) and after (green) application of LY382884 (10 µM). Graph: Representative cell (red) and pooled data (blue). (d) Typical fluorescence transients recorded in line scanning mode before (black) and after (green) co-application of LY367385 (3 µM) and MPEP (3 µM). Graph: Representative cell (red) and pooled data (blue).

Ca^{2+} were not linked to the duration of recording but were specifically associated with the activation of KARs and mGlu receptors and were dependent upon Ca^{2+} release from intracellular stores.

These results demonstrate that GluK1-containing KARs can act together with group I mGlu receptors to modulate calcium signalling within individual giant mossy fibre boutons. The schematic presented in Fig. 7 illustrates the proposed mechanisms underlying induction of mossy fibre LTP. Synaptically-released glutamate acts on three different types of presynaptic glutamate receptor that operate both in an AND gate and OR gate fashion. Activation of GluK1-containing KARs results in Ca^{2+} entry via these receptors whereas activation of $mGlu_1$ and $mGlu_5$ receptors leads to the production of IP_3. Ca^{2+} sensitises IP_3 receptors to enable IP_3 to trigger Ca^{2+} release from intracellular stores, which then activates PKA, presumably via Ca^{2+}-sensitive adenylyl cyclase.

Discussion

In the present study we have made the unexpected discovery that mossy fibre LTP requires activation of both a group I mGlu receptor and a KAR. With respect to the mGlu receptor subtype, activation of either $mGlu_1$ or $mGlu_5$ is sufficient. Consequently selective inhibition of either subtype alone does not affect mossy fibre LTP. With respect to the KAR, we can conclude on the basis of previous work that this contains the GluK1 subtype[12,14]. Here we have demonstrated

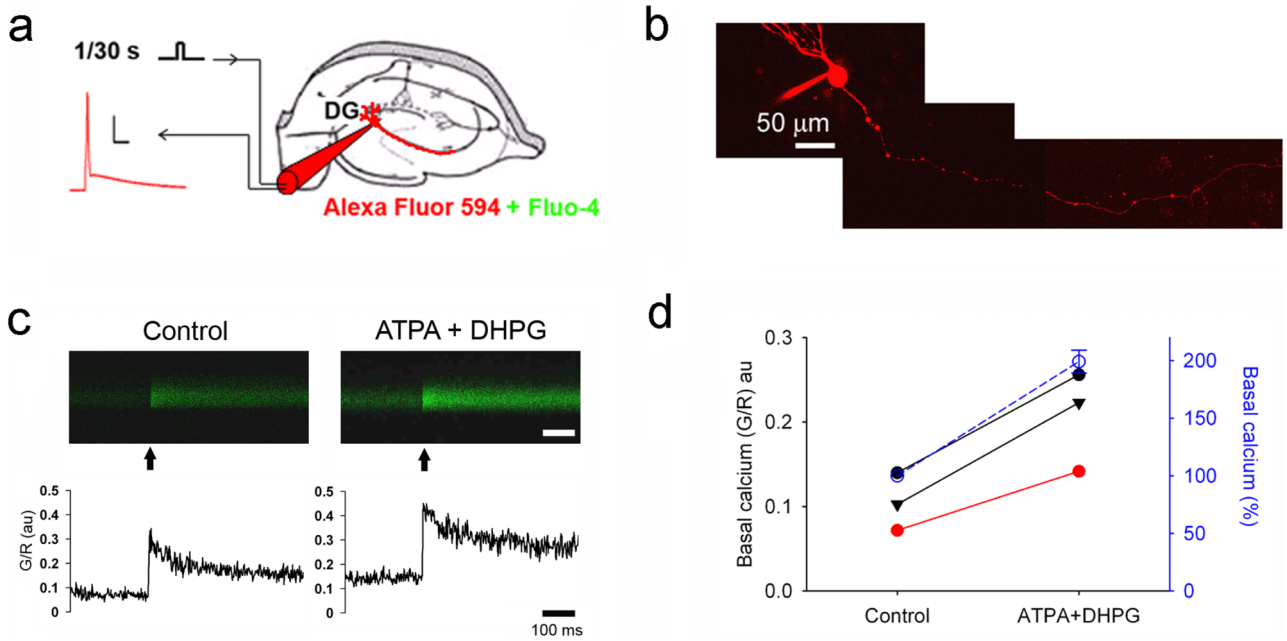

Figure 5 | Chem-LTPm is associated with changes in bouton Ca^{2+} signalling. (a) Schematic of technique: single action potentials were evoked in patched granule cells every 30 s. (b) A representative axon from this set of experiments. (c) Representative fluorescence transients and corresponding traces (averages of 10 successive line scans) before (t ~ 80 min after obtaining a whole cell recording) and after co-application of ATPA (1 µM) and DHPG (3 µM) (t ~ 100 min). White scale bar = 100 ms. (d) Quantified changes in basal calcium following the chem-LTPm protocol: representative bouton (red) and pooled data (blue).

that activation of this KAR, using the highly selective GluK1 agonist ATPA[24], together activation of a group I mGlu receptor is sufficient to induce a stable synaptic potentiation. This chem-LTPm resembles conventional mossy fibre LTP in that it requires activation of PKA[25] and also involves ryanodine-sensitive Ca^{2+} stores[26]. The simplest explanation is that activation of these two receptors alone is sufficient to induce mossy fibre LTP.

There is controversy regarding the role of mGlu receptors in mossy fibre LTP. We and others have reported that MCPG blocks its induction[5,7,27] whereas others have reported no effect[11]. Hitherto the identity of the subtype(s) of mGlu receptor involved in mossy fibre LTP was unknown. Here we identify a role of the group I subtype, which can account for the effects of MCPG. A surprising observation is that the induction of mossy fibre LTP was fully blocked by the combination of a selective $mGlu_1$ and a selective $mGlu_5$ receptor antagonist, whilst blockade of either receptor alone (using a ten-fold higher concentration of antagonist) was ineffective. The failure of either antagonist alone to block mossy fibre LTP is consistent with studies using $mGlu_1$[11] and $mGlu_5$[28] knockouts. The finding that the antagonists applied together are effective demonstrates that, firstly, during the induction of mossy fibre LTP both $mGlu_1$ and $mGlu_5$ receptors are activated synaptically and, secondly, that activation of either one alone is sufficient for the mGlu receptor-mediated signalling event that is required for induction. Why a single presynaptic structure expresses two subtypes of a receptor that can each individually serve the same function is unknown. However, it is likely that under certain conditions both mGlu receptor subtypes may be required, since a partial impairment of mossy fibre LTP was observed in one study using $mGlu_1$ receptor knockout mice[29].

The controversy surrounding the role of mGlu receptors is paralleled with a controversy surrounding the role of KAR, particularly those containing the GluK1 subunit, in mossy fibre LTP. We have reported that a series of GluK1-selective antagonists, including LY382884[12], UBP296[22], 44a[23], later named UBP302[30] and ACET[19], inhibit the induction of mossy fibre LTP and that there is a strong correlation between the potency of compounds as GluK1 antagonists and as inhibitors of mossy fibre LTP over a 100,000-fold concentration range[31]. Thus, it is highly unlikely that off target effects can account for the actions of these GluK1 antagonists as blockers of mossy fibre LTP. On the other hand, studies from knockout mice implicate a role for GluK2 and GluK3 –containing KARs but not GluK1-containing KARs[13,17]. This might be explained in part by functional compensation for the lack of GluK1 receptors throughout development[26,32] and by the involvement of KARs comprising heteromeric assemblies containing GluK1 and other subunits, such as GluK2 and GluK3. However this is unlikely to be the entire explanation. For example, Breustedt & Schmitz (2004) failed to identify a functional role for GluK1 at CA3 synapses, using the GluK1 subunit selective antagonist LY382884.

It has been shown that the recording conditions may also determine the involvement of GluK1-containing KARs in mossy fibre LTP. In particular, while LY382884 invariably blocked LTP in a physiological Ca^{2+} concentration (2 mM) it consistently failed to block LTP in an elevated Ca^{2+} concentration (4 mM), due to the existence of a parallel pathway involving L-type voltage-gated Ca^{2+} channels that was engaged when high Ca^{2+} solutions were employed[26]. Whether a similar compensation accounts for the lack of effect of MCPG in some previous studies is not known, though it is noteworthy that an elevated Ca^{2+} concentration was used in these negative studies[10]. However, this cannot be the only explanation, at least for the differences in the involvement of KARs, since in one study mossy fibre LTP was insensitive to LY382884 even in 2 mM Ca^{2+}[16].

An explanation to reconcile these differences is that there are multiple forms of mossy fibre LTP, with different induction properties. This is not an unlikely scenario. It has already been demonstrated that there are multiple forms of plasticity made between mossy fibres and inhibitory interneurons, which comprise the major targets numerically[33-35]. The properties that we observe for mossy fibre LTP, notably a role for Ca^{2+}-induced Ca^{2+} release[26] and GluK1-containing KARs[12,19,22,23], is paralleled by properties of synaptic facilitation that we observed at these same synapses[14,26]. The

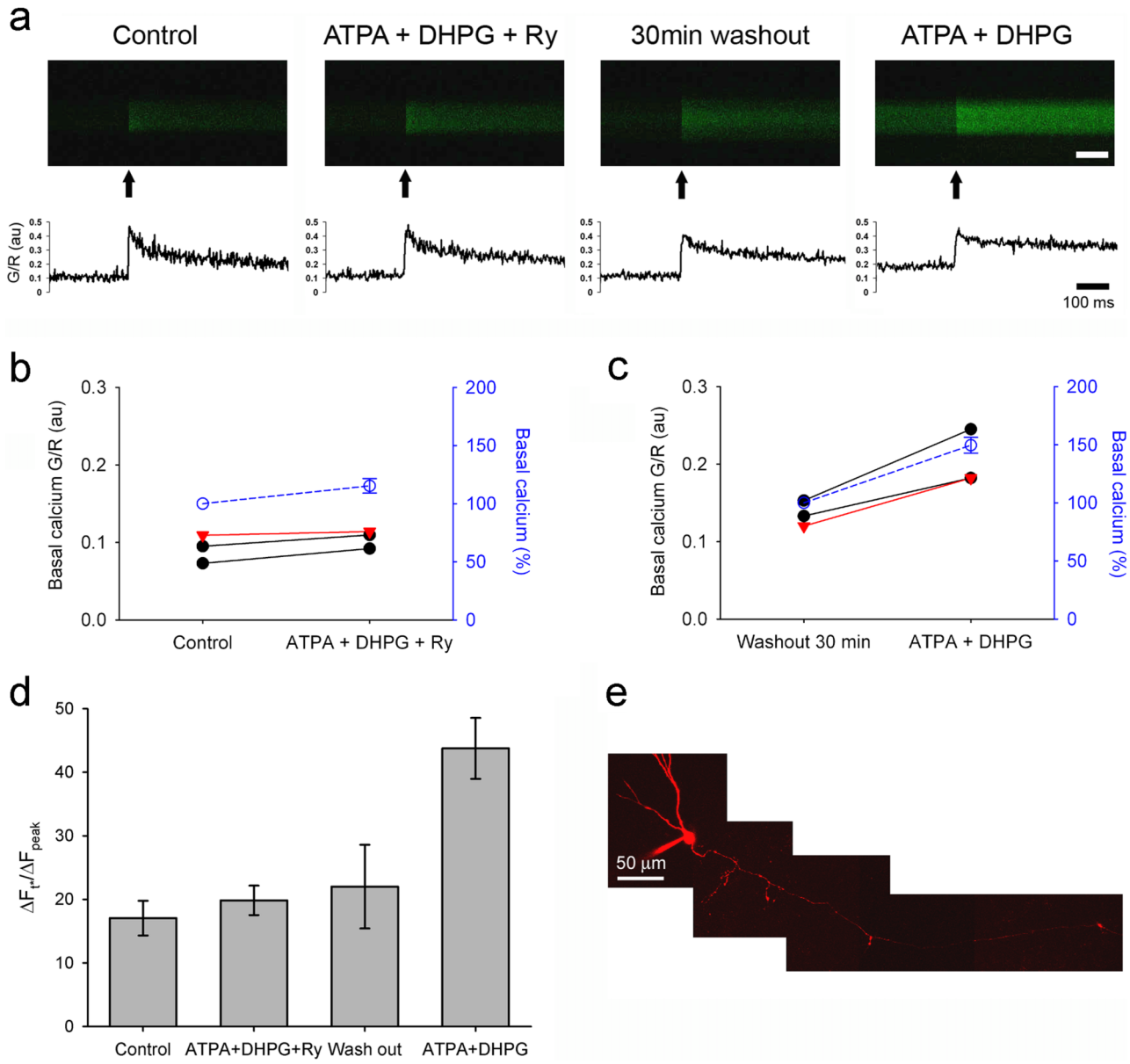

Figure 6 | Evidence for a role of bouton calcium stores in chem-LTPm. (a) A representative experiment showing fluorescence transients in control conditions (t ~ 70 min), following the chem-LTPm protocol in the presence of ryanodine (10 μM) (t ~ 80 min), following washout of ryanodine (t ~ 115 min) and after re-application of ATPA + DHPG (t ~ 135 min). (b) The chem-LTPm protocol did not significantly affect basal Ca^{2+} in the presence of ryanodine. (c) The chem-LTPm protocol increased basal Ca^{2+} following washout of ryanodine. (d) Rate of decay of fluorescence transients. (e) Representative cell from this set of experiments.

prediction that these mechanisms exist on mossy fibre synapses made onto CA3 pyramidal neurons was confirmed by direct measurements from mossy fibre boutons. Thus, it was shown that inhibition of either Ca^{2+} stores[18] or GluK1-containing KARs[19,36] reduced Ca^{2+} transient during synaptic facilitation and that this effect was observed at the giant mossy fibre synapses made onto pyramidal neurons but not the filipodia synapses made with interneurons[18,36]. Therefore, we propose that the mechanism we describe is restricted to one presynaptic structure, the giant mossy fibre synapse, by which the principal cells within the dentate gyrus and CA3 region of the hippocampus communicate.

Having found that activation of either group I mGlu receptors or GluK1-containing KARs is necessary for mossy fibre LTP we wanted to determine whether activation of either alone, or both together, was sufficient to induce mossy fibre LTP, or whether additional factors are also required. The finding that activation of either $mGlu_1$ or $mGlu_5$ could induce LTP provided that GluK1-containing KARs were co-activated suggests that these two receptor systems are sufficient for the induction of mossy fibre LTP. Thus, whilst other neurotransmitter systems may well be able to modulate mossy fibre LTP[37,38], L-glutamate acting via one type of metabotropic plus one type of ionotropic glutamate receptor probably represents the primary trigger for the process.

The mechanisms underlying the induction of mossy fibre LTP are highly controversial. Differences in the results from various laboratories are generally attributed to different pathways being activated, which is a possible scenario given the extensive network of recurrent collaterals within the CA3 region of the hippocampus[39]. We tried to maximise our chances of recording the mossy fibre connection made between dentate gyrus granule cells and CA3 pyramidal neurons by stimulating within the hilus of the dentate gyrus and using stimulus intensity subthreshold for generating significant firing in the CA3 region. Our findings made several predictions about the properties of giant fibre boutons; namely that they should

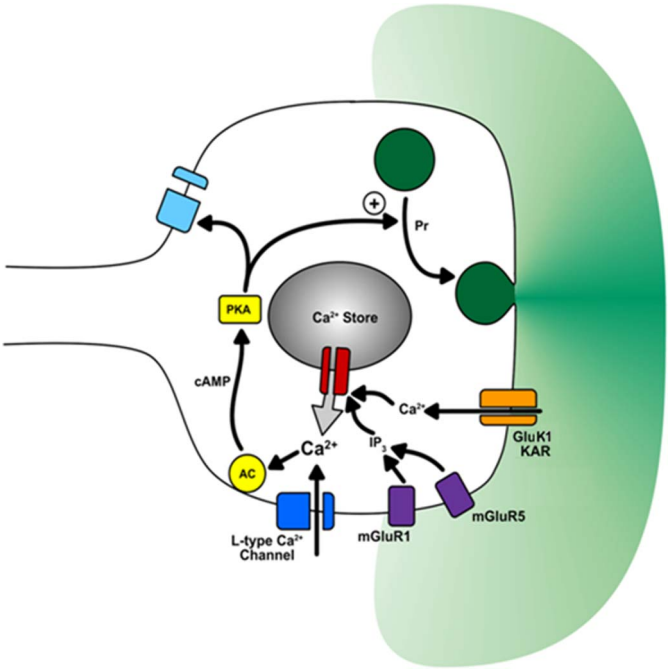

Figure 7 | A proposed mechanism for the induction of mossy fibre LTP. See text for explanation.

possess (or be directly regulated by) GluK1-containing KARs, group I mGlu receptors and ryanodine-sensitive Ca^{2+} stores. By filling individual granule cells with a morphological marker and a Ca^{2+} indicator it is possible to make measurements of Ca^{2+} within identified mossy fibre boutons[18]. As described above, using this approach it has been shown that mossy fibre giant boutons that synapses onto pyramidal neurons, but not the smaller connections onto interneurons, do indeed possess GluK1-containing KARs and ryanodine-sensitive Ca^{2+} stores[18,36]. In the present study we have extended this work to show that Ca^{2+} signalling in these terminals is also regulated by group I mGlu receptors and that all three receptors classes (kainate, mGlu and ryanodine) regulate Ca^{2+} signalling in the giant mossy fibre boutons in slices from young adult rats. This regulation is fully consistent with their predicted role in mossy fibre LTP. Indeed, whilst always possible to construct alternative scenarios, any other explanation for these findings seems highly unlikely.

So why are two types of receptor required for this process? We speculated that this is because group I mGlu receptors are required to generate IP_3 and GluK1-containing KARs to provide a Ca^{2+} signal, which act synergistically to release Ca^{2+} from intracellular stores within presynaptic terminals (Fig. 7). Thus, both Ca^{2+} facilitation and LTP at mossy fibre synapses have an unusual induction mechanism involving three distinct glutamate receptor subtypes that operate both in an AND gate and OR gate fashion. Our Ca^{2+} imaging results suggest further that activation of this receptor combination results in a persistent rise in Ca^{2+} in mossy fibre boutons. This persistent increase may be caused by a corresponding depolarisation of the terminals, as suggested by an alteration in the ability of potassium to depolarise mossy fibre boutons following the induction of LTP[14,40,41]. The increase in terminal Ca^{2+} could then enable subsequent action potentials to trigger more neurotransmitter release, a mechanism entirely consistent with the general observation that mossy fibre LTP is due to an increase in probability of release.

Methods

Preparation of hippocampal brain slices. All animal experiments were carried out in accordance with the UK Scientific Procedures Act, 1986 and associated guidelines. Animals were housed in a regulated environment (21 ± 1 °C) with a 12 h light dark cycle, and food was available *ad libitum*. P28-35 female wistar rats, killed by cervical dislocation, were used for both field recording and calcium imaging experiments. For the field recordings, experiments were performed on parasagittal hippocampal slices (400 μm) using standard techniques[26]. Tissue was cut in ice-cold artificial cerebrospinal fluid (aCSF) containing (mM): NaCl (124), D-glucose (10), $NaHCO_3$ (26), KCl (3), NaH_2PO_4 (1.25), $CaCl_2$ (2) and $MgSO_4$ (1) saturated with 95% O_2 and 5% CO_2. After a recovery period of approximately 60 min, slices were transferred to an interface recording chamber, maintained at 29–31 °C and perfused with aCSF at a rate of ~2 ml/min. For the 2-photon calcium imaging experiments, 400–500 μm slices were prepared using a method that involves isolating the hippocampi and cutting transverse slices at a slight angle (~20°) to optimise the presence of intact mossy fibres[18]. Slices were cut in 'high-magnesium' aCSF and warmed to aid recovery as described previously[19].

***In vitro* extracellular recordings.** Field potential recordings were made using microelectrodes containing 4M NaCl. Synaptic responses were evoked by stimulation of the dentate granule cell layer (mossy fibre pathway) at 0.033 Hz. The presence of synaptic facilitation was established at the beginning of the experiment to confirm that responses were mossy fibre in origin[14], and stimulation intensity was adjusted so that basal fEPSP amplitude was 30 – 40% of maximum. LTP was induced in the presence of D-AP5 (50 μM) by delivering a single tetanus (100 Hz, 1 s) or by using a chemical LTP protocol. Data were collected and analysed on-line using the LTP program: www.ltp-program.com[42]. Data are expressed as mean ± s.e.m. All data were normalised to the baseline preceding any drug application and statistical significance was assessed using the Student's *t*-test. All compounds were obtained from Tocris Cookson (Bristol, UK).

2-photon microscopy and pre-synaptic calcium imaging. Simultaneous 2-photon excitation fluorescence imaging and whole-cell current-clamp electrophysiology experiments were performed as described previously[19]. Briefly, individual dentate granule cells, held at -80 mV, were patch-loaded with a morphological marker, Alexa Fluor 594 (20 μM), and a high affinity calcium indicator, Fluo-4 (200 μM). The patch pipette also contained (mM): K-methanesulphonate (150), KCl (5), HEPES (10), MgATP (3) and NaGTP (0.4) (pH 7.2; 285 mOsm). Whilst recording, slices were perfused continually with aCSF (mM): NaCl (124), KCl (3), $NaHCO_3$ (26), NaH_2PO_4 (1.4), $CaCl_2$ (2), $MgSO_4$ (1), glucose (10) (saturated with 95% O_2 / 5% CO_2), additionally containing PTX (100 μM), CGP-55845 (5 μM) and L-689560 (5 μM). All experiments were performed using a BioRad Radiance 2100 optically linked to a Spectra Physics Mai Tai femtosecond pulsed laser, integrated with an infrared DIC patch clamp set-up[19]. Fluorophores were excited at 810 nm and single axons were traced from the soma into the *stratum lucidum* using the Alexa emission channel (with laser adjusted for optimal emission). Action potentials were evoked by brief somatic command voltage pulses and pre-synaptic calcium transients were recorded in individual giant mossy fibre boutons in line-scan mode at 500 Hz (inter-sweep interval 30 s - 1 min). It took approximately 60 min from first obtaining a whole-cell recording before boutons could be visualised adequately for imaging and in many cases the axons did not extend into the CA3 region of the slice and so the recordings were discarded. Experiments then took a further 30 – 90 min to conduct and in many cases the quality of the recordings deteriorated during this time. As a result it was impossible to obtain large *n* values for each experiment; however, all neurons that were included past strict selection criteria in terms of the quality of the whole-cell recording, the resolution of the imaging and the baseline stability of the bouton Ca^{2+}. Analysis was performed as described previously[19] and data are expressed as mean ± s.e.m. The rate of decay of fluorescent transients was calculated by dividing ΔF at 1.4 s post peak by peak ΔF, these values are expressed as a percentage. Fluorescent dyes were purchased from Invitrogen (Paisley, UK) and all other compounds were obtained from Tocris Cookson (Bristol, UK).

1. Bliss, T. V. & Collingridge, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39 (1993).
2. Collingridge, G. L., Kehl, S. J. & McLennan, H. Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol.* **334**, 33–46 (1983).
3. Harris, E. W. & Cotman, C. W. Long-term potentiation of guinea pig mossy fibre responses is not blocked by N-methyl D-aspartate antagonists. *Neurosci. Lett.* **70**, 132–137 (1986).
4. Nicoll, R. A. & Malenka, R. C. Contrasting properties of two forms of long term potentiation in the hippocampus. *Nature* **377**, 115–118 (1995).
5. Bashir, Z. I. *et al.* Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature* **363**, 347–350 (1993).
6. Kapur, A., Yeckel, M. F., Gray, R. & Johnston, D. L-Type calcium channels are required for one form of hippocampal mossy fibre LTP. *J. Neurophysiol.* **79**, 2181–2190 (1998).
7. Yeckel, M. F., Kapur, A. & Johnston, D. Multiple forms of LTP in hippocampal CA3 neurons use a common postsynaptic mechanism. *Nat. Neurosci.* **2**, 625–633 (1999).
8. Itoh, S. *et al.* Neuronal plasticity in hippocampal mossy fibre-CA3 synapses of mice lacking the inositol-1,4,5-trisphosphate type 1 receptor. *Brain Res.* **901**, 237–246 (2001).
9. Suzuki, E. & Okada, T. Group I metabotropic glutamate receptors are involved in TEA-induced long-term potentiation at mossy fibre-CA3 synapses in the rat hippocampus. *Brain Res.* **1313**, 45–52 (2010).

10. Manzoni, O. J., Weisskopf, M. G. & Nicoll, R. A. MCPG antagonizes metabotropic glutamate receptors but not long-term potentiation in the hippocampus. *Eur. J. Neurosci.* **6**, 1050–4 (1994).
11. Hsia, A. Y. *et al.* Evidence against a role for metabotropic glutamate receptors in mossy fibre LTP: the use of mutant mice and pharmacological antagonists. *Neuropharmacology* **34**, 1567–1572 (1995).
12. Bortolotto, Z. A. *et al.* Kainate receptors are involved in synaptic plasticity. *Nature* **402**, 297–301 (1999).
13. Contractor, A., Swanson, G. & Heinemann, S. F. Kainate receptors are involved in short- and long-term plasticity at mossy fibre synapses in the hippocampus. *Neuron* **29**, 209–216 (2001).
14. Lauri, S. E. *et al.* A critical role of a facilitatory presynaptic kainate receptor in mossy fibre LTP. *Neuron* **32**, 697–709 (2001).
15. Schmitz, D., Mellor, J., Breustedt, J. & Nicoll, R. A. Presynaptic kainate receptors impart an associative property to hippocampal mossy fibre long-term potentiation. *Nat. Neurosci.* **6**, 1058–1063 (2003).
16. Breustedt, J. & Schmitz, D. Assessing the role of GLUK5 and GLUK6 at hippocampal Mossy fibre synapses. *J. Neurosci.* **24**, 10093–10098 (2004).
17. Pinheiro, P. S. *et al.* GluR7 is an essential subunit of presynaptic kainate autoreceptors at hippocampal mossy fibre synapses. *PNAS* **104**, 12181–6 (2007).
18. Scott, R. & Rusakov, D. A. Main determinants of presynaptic Ca2+ dynamics at individual mossy fibre-CA3 pyramidal cell synapses. *J. Neurosci.* **26**, 7071–81 (2006).
19. Dargan, S. L. *et al.* ACET is a highly potent and specific kainate receptor antagonist: characterisation and effects on hippocampal mossy fibre function. *Neuropharmacology* **56**, 121–30 (2009).
20. Schoepp, D. D., Jane, D. E. & Monn, J. A. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* **38**, 1431–1476 (1999).
21. Fitzjohn, S. M. *et al.* The potent mGlu receptor antagonist LY341495 identifies roles for both cloned and novel mGlu receptors in hippocampal synaptic plasticity. *Neuropharmacology* **37**, 1445–1458 (1998).
22. More, J. C. *et al.* Characterisation of UBP296: a novel, potent and selective kainate receptor antagonist. *Neuropharmacology* **47**, 46–64 (2004).
23. Dolman, N. P. *et al.* Synthesis and pharmacology of willardiine derivatives acting as antagonists of kainate receptors. *J. Med. Chem.* **48**, 7867–81 (2005).
24. Clarke, V. R. *et al.* A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. *Nature*, **389**, 599–603 (1997).
25. Weisskopf, M. G., Castillo, P. E., Zalutsky, R. A. & Nicoll, R. A. Mediation of Hippocampal Mossy Fibre Long-Term Potentiation by Cyclic AMP. *Science* **265**, 1878–1882 (1994).
26. Lauri, S. E. *et al.* A role for Ca2+ stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fibre synapses in the hippocampus. *Neuron* **39**, 327–341 (2003).
27. Thompson, K. J., Mata, M. L., Orfila, J. E., Barea-Rodriguez, E. J. & Martinez, J. L., Jr. Metabotropic Glutamate Receptor Antagonist AIDA Blocks Induction of Mossy Fibre-CA3 LTP In Vivo. *J. Neurophysiol.* **93**, 2668–2673 (2005).
28. Lu, Y. M. *et al.* Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* **17**, 5196–205 (1997).
29. Conquet, F. *et al.* Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. *Nature*, **372**, 237–243 (1994).
30. Mayer, M. L., Ghosal, A, Dolman, N. P. & Jane, D. E. Crystal structures of the kainate receptor GluR5 ligand binding core dimer with novel GluR5-selective antagonists. *J. Neurosci.* **26**, 2852–61 (2006).
31. Nisticò, R. *et al.* GLUK1 receptor antagonists and hippocampal mossy fiber function. *Int Rev Neurobiol.* **85**, 13–27 (2009).
32. Kerchner, G. A., Wilding, T. J., Huettner, J. E., Zhuo, M. Kainate receptor subunits underlying presynaptic regulation of transmitter release in the dorsal horn. *J Neurosci.* **22**, 8010–7 (2002).
33. Schmitz, D., Frerking, M. & Schmitz, D., Frerking, M. & Nicoll, R. A. Synaptic activation of presynaptic kainate receptors on hippocampal mossy fiber synapses. *Neuron* **27**, 327–38. (2000)
34. Suzuki, E. & Okada, T. Regional differences in GABAergic modulation for TEA-induced synaptic plasticity in rat hippocampal CA1, CA3 and dentate gyrus. *Neurosci. Res.* **59**, 183–90 (2007).
35. Galván, J., Calixto, E. & Barrionuevo, G. Bidirectional Hebbian plasticity at hippocampal mossy fiber synapses on CA3 interneurons. *J. Neurosci.* **52**, 14042–55 (2008).
36. Scott, R., Lalic, T., Kullmann, D. M., Capogna, M. & Rusakov, D. A. Target-Cell specificity of kainate autoreceptor and Ca^{2+}-store-dependent short-term plasticity at hippocampal mossy fibre synapses. *J. Neurosci.* **49**, 13139–13149 (2008).
37. Maeda, T., Kaneko, S. & Satoh, M. Bidirectional modulation of long-term potentiation by carbachol via M1 and M2 muscarinic receptors in guinea pig hippocampal mossy fibre-CA3 synapses. *Brain Res.* **619**, 324–330 (1993).
38. Kaneko, S., Maeda, T. & Satoh, M. Cognitive enhancers and hippocampal long-term potentiation in vitro. *Behav. Brain Res.* **83**, 45–49 (1997).
39. Kwon, H. B., Castillo, P. E. Long-term potentiation selectively expressed by NMDA receptors at hippocampal mossy fiber synapses. *Neuron* **57**, 108–20 (2008).
40. Barrionuevo, G., Kelso, S. R., Johnston, D. & Brown, T. H. Conductance mechanism responsible for long-term potentiation in monosynaptic and isolated excitatory synaptic inputs to hippocampus. *J. Neurophysiol.* **55**, 540–550 (1986).
41. Schmitz, D., Mellor, J., Nicoll, R. A. Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. *Science* **291**, 1972–1976 (2001).
42. Anderson, W. W. & Collingridge, G. L. The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *J. Neurosci. Methods* **108**, 71–83 (2001).

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Author Contributions

R.N., S.L.D. and M.A. carried out all experiments and analyzed data. G.L.C. and Z.A.B. conceived and designed the study and supervised all the experiments. G.L.C. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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